NFATc2 and T-bet contribute to T-helper-cell-subset-specific regulation of IL-21 expression

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T helper (Th) 2 cells selectively express IL-21 in addition to the classic Th2 cytokines IL-4, IL-5, and IL-13. In contrast to these clustered Th2 cell cytokine genes, the IL-21 gene resides on a different chromosome and is not coordinately regulated by the same locus control region that directs the expression of other Th2 cytokines. We demonstrate that the proximal promoter of IL-21 controls its Th-cell-subset-specific expression through the action of NFATc2 and T-bet. Whereas NFATc2 directly binds to and activates transcription of the IL-21 promoter in Th2 cells, T-bet represses IL-21 transcription by inhibiting the binding of NFATc2 to the promoter in Th1 cells. These data suggest that there are multiple mechanisms by which Th-cell-subset-specific cytokine genes are regulated.

cytokine | promoter | transcription factor

Naïve CD4$^+$ T helper precursor (Thp) cells can differentiate into either Th1 or Th2 cell subsets after activation. Th1 cells produce IFN-γ and direct cell-mediated immune responses against intracellular pathogens, whereas Th2 cells produce IL-4, IL-5, and IL-13 and mediate humoral responses against extracellular pathogens. The cytokine expression profile of each Th cell subset is regulated by cell-specific transcription factors. In Th1 cells, IFN-γ production and Th1 cell lineage commitment is controlled by T-bet (1), whereas in Th2 cells, specific cytokine production is mediated by the transcription factors c-Maf and GATA3 (2–6).

Although ubiquitously expressed, non-lineage-specific transcription factors can also induce subset-specific transcription of cytokine genes. The STATs confer cell-specific gene expression because they are selectively activated through specific cytokine–cytokine receptor interactions (reviewed in ref. 7). NFATc1 and NFATc2 are expressed in both Th1 and Th2 cells and are essential for cytokine gene transcription because T cells deficient in both NFATc1 and NFATc2 are severely impaired in the production of Th1- and Th2-cell-specific cytokines (8). Although these potent factors are nonselectively induced upon stimulation, restricted access *in vivo* to cytokine genes, as a result of local chromatin remodeling, can confer Th-cell-subset-specific expression. For example, NFATc2 was found to bind the IL-4 enhancer and promoter only in stimulated Th2 cells, whereas it bound the IFN-γ promoter only in activated Th1 cells (9).

The Th2-lineage-specific cytokines genes IL-4, IL-5, and IL-13 are clustered in a 120-kb region on chromosome 11 in the mouse (10, 11). Their expression is thought to be coordinately regulated by changes in chromatin accessibility and by several positive regulatory regions located within the Th2 cell cytokine locus, including the conserved noncoding sequence 1 (CNS-1), whose deletion diminishes the capacity to produce all three Th2 cell cytokines, and a 3′ distal enhancer of the IL-4 gene that becomes accessible to NFAT only in antigen-stimulated Th2 cells (9, 12, 13). In addition, a locus control region has been recently identified that promotes IL-4 and IL-13 transcription in an integration-site-independent and copy-number-dependent manner (14). It is clear that the coordinated expression of Th2-cell-lineage-specific cytokines involves the interplay of different regulatory sequences, chromatin dynamics, and lineage-specific transcription factors.

IL-21 is a recently described cytokine that has been found to have a diverse array of effects on B, T, dendritic, and NK cells. IL-21 was originally found to be a product of activated CD4$^+$ T cells (15), although further investigation has revealed that it is selectively expressed by Th2 cells *in vitro* and *in vivo* (16). Like IL-4, IL-21 mRNA is detectable at very low levels in Thp cells after primary stimulation and is greatly induced upon secondary stimulation of developing Th2 cells. Interestingly, the IL-21 gene is not located within the same locus that coordinately regulates Th2-cell-specific expression of IL-4, IL-5, and IL-13 but, rather, lies on a different chromosome entirely (murine chromosome 3). Thus, IL-21 expression is likely to be regulated independently of the other classic Th2 cell cytokines, and the mechanism of Th2-cell-specific regulation of IL-21 remains to be understood. In this report, we have identified and cloned a functional murine IL-21 proximal promoter. Combining promoter analysis and genetic experiments, our data demonstrate that IL-21 is regulated by the Th-cell-subset-specific action of two transcription factors, NFATc2 and T-bet. NFATc2 is important in promoting the expression of IL-21 in Th2 cells, and T-bet represses IL-21 expression in Th1 cells.

Materials and Methods

Initial Isolation and Characterization of the IL-21 Promoter. Genomic sequence upstream of the first coding exon of the IL-21 gene was retrieved by using the Celera Discovery System (www.celera.com). Putative transcription factor binding sites were determined by using the web-based software MATINSPECTOR (www.genomatix.de) and RVISTA 2.0 (http://rvista.dcode.org) (17, 18). The transcriptional start site was mapped by 5′ RACE with the First Choice RLM-RACE kit (Ambion) and mRNA from Th2-skewed cells restimulated on day 7 with 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28.

Plasmid Construction and Mutagenesis. The 267-bp promoter region was amplified by PCR of genomic DNA from 129 mice using Platinum *Taq* (Invitrogen), sequenced, and cloned into the *Kpn*I and *Xho*I sites of pGL2 (Promega) to generate the IL-21-luc and reverse IL-21-luc constructs. Oligonucleotides used for genomic PCR were 5′-CCCTTGTGAATGCTGAAAACTG-3′ and 5′-GGCCTTGGTCTGGTTCTCACT-3′. For details of site-directed matagenesis, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Abbreviations: ChIP, chromatin immunoprecipitation; PMA+I, PMA and ionomycin; Th, T helper; Thp, Th precursor.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY901991).

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¶L.H.G. has equity in and is on the Corporate Board of Bristol-Myers Squibb, and she has equity in and is a paid consultant for Healthcare Ventures SAB and Mannkind Corporation, a biopharmaceutical company focused on the development and commercialization of treatments for diseases including cancer and autoimmune diseases.

Mice and Cell Lines. Mice 6–8 weeks of age were used in all experiments. C57BL/6 mice were obtained from Taconic Farms, and BALB/c mice were obtained from The Jackson Laboratory. NFATc2$^{-/-}$ (19) and IFN-$\gamma^{-/-}$ T-bet$^{-/-}$ mice on the BALB/c background and T-bet$^{-/-}$ mice (20) on the C57BL/6 background were obtained from Laurie H. Glimcher. All cell lines and primary cells were maintained in complete medium containing RPMI medium 1640 as described in refs. 16 and 21.

Luciferase Reporter-Gene Assays. Cell lines were transfected as described in ref. 21 by using 5×10^6 cells and 10 μg each of reporter and expression plasmids for M12 and EL4 cells, or 1×10^7 cells and 20 μg of each plasmid for D10 and AE7 cells. Each transfection was split into two cultures, rested for 6 h, and then stimulated with either 50 ng/ml PMA and 1 μM ionomycin (PMA+I) or 1 μg of anti-CD3 for 12 h before cell harvest. NIH 3T3 cells were transfected with 0.5 μg of each plasmid by using Effectene (Qiagen) and stimulated with PMA+I for 12 h before harvest. All cells were cotransfected with 5 ng of pRL-TK (Promega) as a normalization control for transfection efficiency. Luciferase assays were performed by using the Dual-Luciferase Reporter Assay System (Promega). Expression plasmids NFATc2 (pEFBOS), p65 (pMEX), and T-bet (pcDNA3.1) and promoter constructs NFAT2×-luc and NF-κB-luc were obtained from Laurie H. Glimcher.

CD4$^+$ T Cell Purification, Differentiation, and RNA Analysis. Naïve CD4$^+$ T cells were purified by cell sorting to 95–98% purity for *in vitro* differentiation cultures, as described in ref. 16. Cells were restimulated on day 6 with anti-CD3/CD28 for 16 h. RNA was isolated, and cytokine production was determined by real-time PCR as described in ref. 16. Primers and TaqMan probes for IL-21, IL-4, IFN-γ, β-actin, and T-bet are published in refs. 16 and 22.

Retroviral Transduction of CD4$^+$ T cells. Retroviral transduction of primary BALB/c or IFN-$\gamma^{-/-}$ T-bet$^{-/-}$ CD4$^+$ T cells was performed as described in ref. 1. Cells were expanded and maintained under Th2 cell conditions, and GFP-positive cells were sorted on day 14. Cells were restimulated on day 15 with anti-CD3/CD28 for 12 h. Cytokine and T-bet expression was analyzed by real-time PCR.

Chromatin Immunoprecipitation (ChIP). Chromatin samples were isolated from naïve CD4$^+$ T cells that were skewed under Th1 or Th2 conditions for 14 days and then restimulated with PMA+I for 2–3 h. The ChIP procedure was carried out as described in ref. 23, using antibodies to RNA polymerase II (N-20, Santa Cruz Biotechnology), acetyl histone H3 lysine 9 (designated as H3AcK9; 06-942, Upstate), T-bet (H-210, Santa Cruz Biotechnology), NFATc2 (T2B1, courtesy of Anjana Rao, Harvard Medical School), and NFATc1 (7A6, Santa Cruz Biotechnology). Significant positive signals were never observed in 7A6-precipitated samples for any promoter tested (Fig. 3*D* and data not shown). Therefore, because of the lack of a positive control, 7A6 can only be used to assess background signals for nonspecific interactions. Primers for all promoters tested are located within the proximal promoter region.

Coimmunoprecipitation and Immunoblotting. 293T cells were transfected by using Effectene with 1 μg of NFATc2 (pEFBOS), T-bet (pcDNA 3.1), or both. Cells were stimulated for 2–3 h with

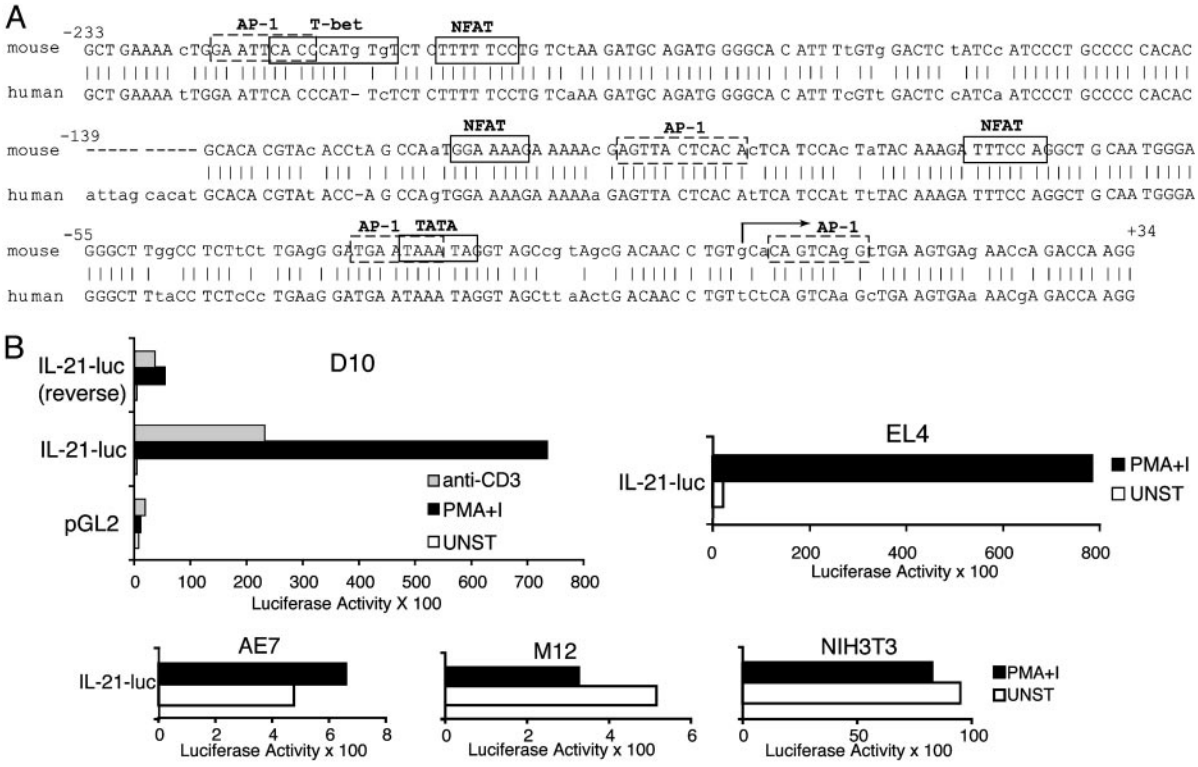

Fig. 1. Selective expression of IL-21 by Th2 cells is directed by the IL-21 proximal promoter. (*A*) Comparison of the genomic sequence of murine and human IL-21 promoter regions. The transcription initiation site is designated +1. Potential TATA box and transcription factor binding sites that met the most stringent requirements, using MATINSPECTOR and RVISTA 2.0 analysis, are indicated and boxed (18). (*B*) D10, EL4, AE7, M12, and NIH 3T3 cells were transfected with either pGL2, IL-21-luc, or IL-21-luc (reverse). Cells were left to rest 6 h and then left unstimulated (UNST) or stimulated with PMA+I or anti-CD3 for 12 h before luciferase activity was measured. Luciferase activity is expressed relative to the expression of a cotransfected *Renilla* luciferase promoter (pRL-TK) to control for transfection efficiency. The results shown are representative of four independent experiments.

PMA+I and then harvested in whole-cell lysate buffer, as described in ref. 16. T-bet was immunoprecipitated by using 1 μg of T-bet antibody (4B10, Santa Cruz Biotechnology), and immunoprecipitates were run on SDS/PAGE and immunoblotted, as described in ref. 16, with anti-NFATc2 (46G65, Santa Cruz Biotechnology).

Results

Cloning and Characterization of the Murine IL-21 Proximal Promoter. To understand what factors confer Th2 cell-type specificity of IL-21 expression, we sought to identify and characterize the murine IL-21 proximal promoter. By comparing the genomic sequences of the murine and human IL-21 loci for highly conserved noncoding sequences, we identified a 267-bp region located immediately 5′ of the first coding exon that is 88% conserved between mouse and human (Fig. 1*A*). Sequence analysis of this region for putative transcription factor binding sites revealed the presence of a TATA box, three NFAT binding sites, several AP-1 consensus sites, and a T-bet consensus site (Fig. 1*A*). The transcription start site of the IL-21 gene was mapped to this region by 5′RACE and is indicated (arrow) as position +1 in Fig. 1*A*.

To determine whether this portion of the IL-21 gene is capable of regulating transcription, the conserved region (−245/+36) was cloned upstream of a luciferase reporter gene (IL-21-luc), and its ability to activate transcription was evaluated by transfection of a variety of cell lines. Basal activity of the promoter is very minimal; however, it is highly inducible by PMA+I stimulation in both D10 cells, a murine Th2 clone, and in EL4 cells, a murine T lymphoma that produces IL-4 and IL-2 upon activation (Fig. 1*B*). In addition, TCR engagement using anti-CD3 was also able to induce IL-21 promoter activity in D10 cells. An inverted IL-21 promoter lacked the ability to activate reporter-gene transcription upon stimulation. Moreover, promoter activity was not induced by PMA+I stimulation in AE7 (Th1 clone), M12 (B lymphoma), or NIH 3T3 (fibroblast) cells, suggesting that both the cell type and the Th cell subset specificity of IL-21 gene expression reside within the 267-bp region cloned as its proximal promoter.

NFATc2 Activates IL-21 Transcription. Given that IL-21 promoter activity is strongly induced through TCR signaling and that three potential NFAT binding sites were identified, we investigated whether NFAT plays a role in regulating IL-21 gene expression. EL4 cells were transfected with IL-21-luc alone or with an expression vector for NFATc2, and the luciferase activity of both unstimulated and PMA+I-stimulated cells was measured. Without stimulation, IL-21-luc activity was induced 40-fold over its basal activity in the presence of exogenous NFATc2 (Fig. 2*A*). PMA+I stimulation of NFATc2-transfected cells increased the level of IL-21-luc activity to >600-fold over its basal activity, suggesting that although NFATc2 could by itself transactivate the IL-21 promoter, other factors that are induced during PMA+I treatment, such as AP-1 family members, can further augment promoter activity. Although NFATc2 is a potent activator of the IL-21 promoter, neither NFATc1 nor NFATc3 transactivated the IL-21-luc reporter-gene construct (data not shown), suggesting that NFATc2 is the preferred NFAT family member regulating IL-21 gene expression. Site-directed mutagenesis of the NFAT site at −118/−113 in the IL-21-luc reporter construct ablates its PMA+I-inducible activity, and mutation of the site at −203/−197 also diminishes PMA+I-inducible activity, although not as severely. Mutation of both NFAT sites results in the total loss of PMA+I-inducible activity (Fig. 5*A*, which is published as supporting information on the PNAS web site).

To show that NFAT activation is required for endogenous IL-21 expression, we treated Th2-skewed cells with cyclosporin A and found that, like IL-4 and IFN-γ gene expression, IL-21 transcription is also abrogated by the addition of cyclosporin A (Fig. 5*B*). To understand the specific role of NFATc2 during IL-21 gene transcription, we examined IL-21 production under Th2-cell-skewing conditions of NFATc2$^{-/-}$ Thp cells. Upon restimulation of NFATc2$^{-/-}$ Th2 cells with anti-CD3/CD28, we found that IL-21 production was dramatically reduced compared with WT Th2 cells, although not completely diminished, suggesting that NFATc2 is required for the optimal production of IL-21 in Th2 cells (Fig. 2*B*).

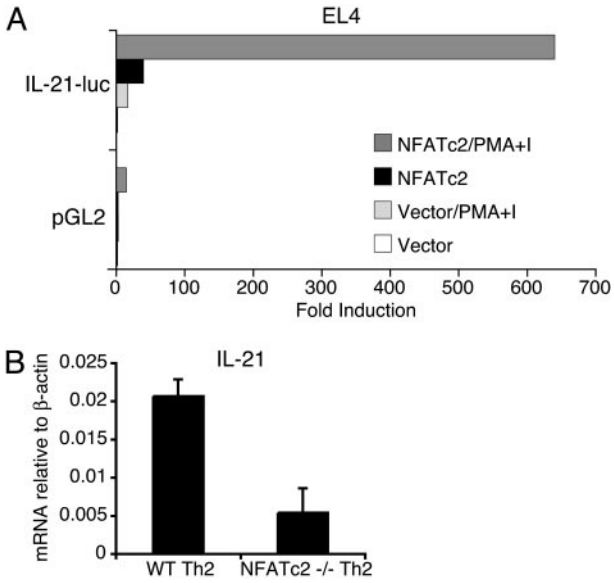

Fig. 2. NFATc2 promotes transcriptional activity of the IL-21 proximal promoter. (*A*) EL4 cells were transfected with either the pGL2 or IL-21-luc and either pEFBOS or pEFBOS expressing NFATc2. Reporter-gene assays were preformed as described in Fig. 1*B*. Activity is expressed as the fold induction in luciferase activity relative to unstimulated IL-21-luc reporter activity and is adjusted for transfection efficiency with pRL-TK. The results shown are representative of three independent experiments. (*B*) Naïve Thp cells from NFATc2$^{-/-}$ and BALB/c (WT) mice were cultured under Th2-skewing conditions for 6 days. Cells were restimulated with anti-CD3/28. Cytokine expression was analyzed by real-time PCR and is shown relative to β-actin. The results shown are the average of three independent experiments.

T-bet Represses IL-21 Expression. In addition to the NFAT sites, we also located a putative T-box consensus site (−217/−207) in the IL-21 proximal promoter. T-bet overexpression in Th2 cells induces the expression of significant levels of IFN-γ and results in reduced levels of Th2 cell cytokines like IL-4 and IL-5 (1, 20). To test whether T-bet can affect the IL-21 promoter, we transfected EL4 cells with IL-21-luc alone or with an expression vector for T-bet and measured the luciferase activity of unstimulated and PMA+I-stimulated cells. We found that T-bet overexpression represses the PMA+I-inducible activation of the IL-21-luc reporter construct (Fig. 3*A*). There was no effect of T-bet overexpression on IL-21-luc basal activity in unstimulated cells (data not shown).

To determine what effect T-bet has on endogenous gene expression of IL-21, we analyzed T-bet$^{-/-}$ mice for any differences in IL-21 transcription. Naïve Thp cells were purified from T-bet$^{-/-}$ and WT mice, skewed either under Th1 or Th2 cell conditions for 1 week, and then restimulated with anti-CD3/CD28 and analyzed for cytokine production by real-time PCR. Remarkably, we found that T-bet$^{-/-}$ Th1 cells are capable of producing as much IL-21 as WT Th2 cells (Fig. 3*B*). There is no difference in the expression levels of GATA3 mRNA between

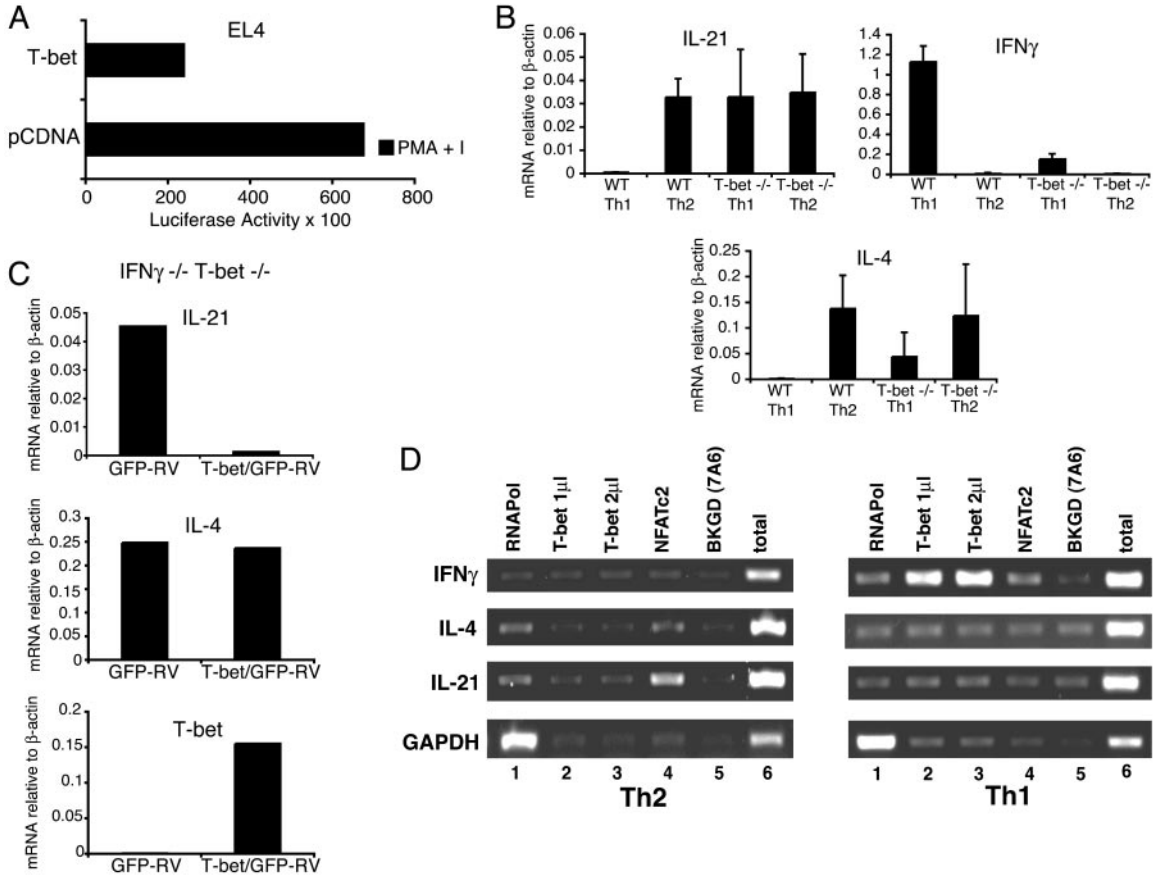

Fig. 3. T-bet represses IL-21 promoter activity and endogenous IL-21 gene expression but does not bind the IL-21 proximal promoter. (*A*) EL4 cells were transfected with the IL-21-luc reporter construct and either pcDNA3.1 or a pcDNA3.1 vector expressing T-bet. Reporter-gene assays were preformed as described in Fig. 1*B*. The results are representative of four independent experiments. (*B*) Naïve Thp cells from T-bet$^{-/-}$ and C57BL/6 (WT) mice were cultured under Th1- and Th2-skewing conditions for 6 days. Cells were restimulated with anti-CD3/28. Cytokine expression was analyzed by real-time PCR and is shown relative to β-actin. The results shown are the average of three independent experiments. (*C*) Ectopic expression of T-bet represses endogenous IL-21 transcription in effector Th2 cells. IFN-$\gamma^{-/-}$ T-bet$^{-/-}$ CD4^{+} T cells were cultured under Th2 skewing conditions for a total of 2 weeks. On day 9 of culture, cells were infected with a retrovirus that either expresses GFP alone (GFP-RV) or coexpresses T-bet and GFP (T-bet/GFP-RV). Cells were expanded and maintained under Th2 culture conditions. GFP-positive cells were purified by flow cytometry on day 14 and restimulated on day 15 with anti-CD3/28 for 16 h. Cytokine and T-bet expression were analyzed by real-time PCR and are shown relative to β-actin. (*D*) Naïve Thp cells were cultured under Th1- or Th2-skewing conditions for 14 days. Cells were restimulated with PMA+I for 2–3 h and fixed before ChIP assays with antibodies to RNA polymerase II (lane 1), T-bet (lanes 2 and 3), NFATc2 (lane 4), or the 7A6 antibody, which represents the background signal (lane 5). The total input control is in lane 6. PCR was performed on the IFN-γ, IL-4, IL-21, and GAPDH promoters as indicated.

T-bet$^{-/-}$ and WT Th1 cells (data not shown). T-bet$^{-/-}$ Th2 cells produced similar amounts of IL-21 and IL-4 as compared with WT Th2 cells. Consistent with previous studies, T-bet$^{-/-}$ Th1 cells are impaired in the production of IFN-γ and also have a slight increase in IL-4 expression as compared with WT Th1 cells (20). Interestingly, the increase in IL-21 production by T-bet$^{-/-}$ Th1 cells was more dramatic than that seen for IL-4 production.

We used retroviral transduction to ectopically express T-bet in effector Th2 cells to determine whether T-bet can repress endogenous IL-21 transcription in differentiated Th2 cells. To rule out any secondary effects of increased IFN-γ levels, the result of ectopic T-bet expression, on IL-21 expression we used IFN-$\gamma^{-/-}$ T-bet$^{-/-}$ CD4^{+} T cells. Cells were skewed under Th2 cell conditions for 9 days before being infected with a retrovirus that expresses either GFP alone (GFP-RV) or T-bet and GFP (T-bet/GFP-RV). Infected cells were maintained under Th2-cell-skewing conditions, sorted for GFP-positive cells on day 14, and analyzed by real-time PCR upon tertiary stimulation with anti-CD3/CD28. Even in the absence of IFN-γ, ectopic expression of T-bet in Th2 cells completely represses IL-21 transcription (Fig. 3*C*). Surprisingly, the lack of IFN-γ relieved the T-bet-mediated inhibition of IL-4 transcription. IL-21 expression was also ablated when WT CD4^{+} T cells were used (data not shown). Consistent with previous studies, we also found that T-bet expression in effector WT Th2 cells induces the transcription of high levels of IFN-γ and leads to the partial inhibition of IL-4 transcription (1). Interestingly, however, the T-bet mediated repression of IL-21 transcription was more dramatic than that of IL-4. This trend is consistent in T-bet$^{-/-}$ Th1 cells, which produce Th2 levels of IL-21 while IL-4 production is only slightly increased (Fig. 3*B*). These data indicate that T-bet is a potent repressor of IL-21 gene expression in Th1 cells, and that NFATc2 and T-bet together could be responsible for the Th cell subset specificity of IL-21 expression.

NFATc2, but Not T-bet, Binds the IL-21 Proximal Promoter *in Vivo*. We next conducted ChIP experiments to establish direct binding of these transcription factors to the IL-21 proximal promoter *in vivo*. ChIP experiments were performed on naïve CD4^{+} T cells skewed under Th1 or Th2 cell conditions for 14 days. Confirming our reporter-gene assays, NFATc2 was found to bind the IL-21 promoter in Th2 cells but not Th1 cells (Fig. 3*D*, lane 4).

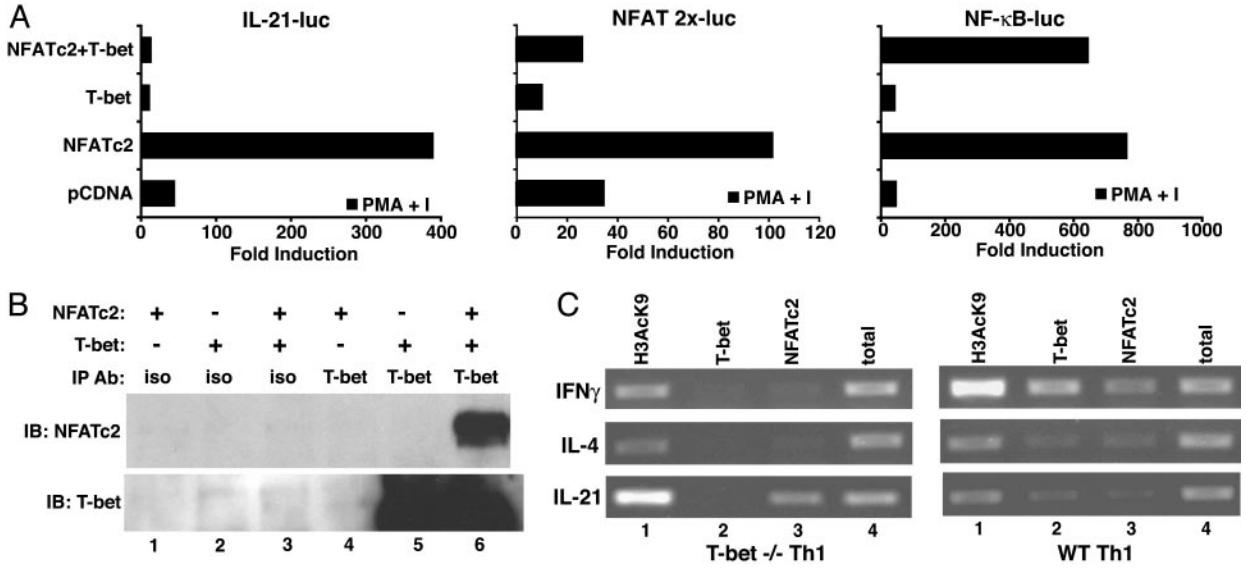

Fig. 4. T-bet prevents NFATc2-mediated activation of IL-21 gene expression. (*A*) EL4 cells were transfected with the appropriate promoter construct (IL-21-luc or NFAT2×-luc) and pcDNA3.1, expression vectors for NFATc2, T-bet, or both NFATc2 and T-bet. To show the specificity of T-bet-mediated repression of NFAT activity, EL4 cells were also transfected with NF-κB-luc promoter construct and pcDNA3.1, expression vectors for p65, T-bet, or both p65 and T-bet. Reporter-gene assays were preformed as described in Fig. 1*B*. Activity is expressed as the fold induction in luciferase activity relative to unstimulated reporter activity and is adjusted for transfection efficiency with pRL-TK. The results are representative of two to three independent experiments. (*B*) 293T cells were transfected with expression vectors for NFATc2, T-bet, or both NFATc2 and T-bet and immunoprecipitated by using T-bet or isotype control antibodies. Proteins were run on SDS/PAGE and immunoblotted with anti-NFATc2 or anti-T-bet. (*C*) Naïve WT or T-bet$^{-/-}$ Thp cells were cultured under Th1-skewing conditions for 14 days. Cells were restimulated with PMA+I for 2–3 h and fixed before ChIP assays by using antibodies to acetylated histone H3 (H3AcK9, lane 1), T-bet (lane 2), or NFATc2 (lane 3). The total input control is in lane 4. PCR was performed on the IFN-γ, IL-4, and IL-21 promoters as indicated.

Consistent with previous studies (9, 24), NFATc2 also bound to the IL-4 promoter only in Th2 cells and to the IFN-γ promoter only in Th1 cells. Surprisingly, our results indicate that T-bet does not bind the IL-21 proximal promoter in Th1 cells, even though T-bet is capable of binding the IFN-γ promoter in these cells (Fig. 3*D*, lanes 2 and 3). These results suggest that the predicted T-bet consensus site within the IL-21 proximal promoter is not a functional binding site and imply that T-bet is repressing IL-21 expression through a mechanism other than direct binding of the IL-21 proximal promoter.

T-bet Disrupts NFAT-Mediated Transactivation of the IL-21 Promoter. Given that the T-bet-mediated inhibition of the IL-21 promoter was stimulation-dependent in reporter-gene assays (Fig. 3*A*) and also that NFATc2 could not bind the IL-21 promoter in Th1 cells in ChIP experiments (Fig. 3*D*), we examined the effects of T-bet on NFATc2-mediated activation of the IL-21 promoter in reporter-gene assays. EL4 cells were transfected with IL-21-luc and expression vectors for either NFATc2 and/or T-bet, and luciferase activity was measured after PMA+I stimulation. The repressive effect of T-bet on IL-21 promoter activity was dominant over the large activation normally seen with NFATc2 (Fig. 4*A Left*), indicating that T-bet inhibits the activity of NFATc2 in the context of the IL-21 promoter. These results are consistent with the experiments in which ectopic expression of T-bet is able to ablate IL-21 expression in effector Th2 cells (Fig. 3*C*). Even in the context of a basic NFAT promoter element (NFAT2×-luc), lacking T-bet binding sites, T-bet impairs the transactivation of the NFAT2×-luc construct in the presence of PMA+I (Fig. 4*A Center*). However, T-bet does not inhibit the p65-mediated activation of an NFAT-independent promoter such as an NF-κB-luciferase construct (Fig. 4*A Right*).

Because T-bet does not directly bind the proximal IL-21 promoter, one potential mechanism for how T-bet can repress NFATc2-mediated activation is by interacting with NFATc2 and thereby preventing its binding to the IL-21 promoter. To examine whether NFATc2 and T-bet physically interact, we transfected 293T cells with expression vectors for NFATc2 and/or T-bet. Whole-cell lysates from PMA+I-stimulated cells were immunoprecipitated with T-bet or isotype control antibodies and then separated by SDS/PAGE. Immunoblotting with anti-NFATc2 revealed that NFATc2 and T-bet do indeed physically interact (Fig. 4*B*, lane 6). Attempts at demonstrating endogenous interaction have thus far failed, possibly because of the poor quality of available reagents.

Finally, we performed ChIP assays examining NFATc2 binding in WT and T-bet$^{-/-}$ Th1 cells. Interestingly, we found that NFATc2 binds to the IL-21 promoter in T-bet$^{-/-}$ but not WT Th1 cells (Fig. 4*C*, lane 3), whereas T-bet and NFATc2 only bind to the IFN-γ promoter in WT Th1 cells (Fig. 4*C*, lanes 2 and 3). As a positive control for active transcription, we examined histone modifications, specifically for the K9 acetylation of histone H3. We found that H3 was hyperacetylated at the IL-21 promoter in T-bet$^{-/-}$ but not WT Th1 cells (Fig. 4*C*, lane 1). The binding of NFATc2 and acetylated histone H3 to the IFN-γ and IL-4 promoters is very similar to that already published (24). Taken together, these data suggest that the interaction of T-bet and NFATc2 in Th1 cells can play a significant role in the repression of IL-21 gene expression by inhibiting the binding of NFATc2 to the proximal promoter and thus its activation.

Discussion

There is increasing evidence that NFATc2 is a positive regulator of Th2 cytokines (8, 9, 19, 25, 26), and we show here that it is also a potent inducer of IL-21 gene expression. Reporter-gene and ChIP assays demonstrated that NFATc2 directly binds the IL-21 promoter *in vivo*. Also, the endogenous production of IL-21 by Th2 cells in the presence of cyclosporin A or by NFATc2$^{-/-}$ Th2 cells is diminished. Although NFATs are nonselectively induced in both Th1 and Th2 cell subsets, they can still operate in a subset-specific manner by cooperating with other transcription factors with restricted expression, such as c-Maf or GATA3 (4,

24). In addition, there is also evidence of subset-restricted binding of NFATc2 to cytokine regulatory regions as a result of chromatin remodeling during Th-cell differentiation, where NFATc2 binds to the IL-4 promoter only in Th2 cells and to the IFN-γ promoter in Th1 cells (9, 24). Preliminary evidence that the IL-21 promoter can also be transactivated by GATA3 or JunB, an AP-1 transcription factor that selectively accumulates in Th2 cells but not in Th1 cells (27), suggests that these factors may also contribute to the Th2-specific expression of IL-21, possibly in cooperation with NFATc2.

We have also delineated a role for T-bet in negatively regulating IL-21 gene expression. We found that T-bet inhibits the PMA+I-induced activation of the IL-21-luc reporter construct and that the Th2-cell-specific expression pattern of IL-21 is abolished in T-bet$^{-/-}$ mice. In addition, IL-21 gene expression is impaired in effector Th2 cells ectopically expressing T-bet, independent of IFN-γ expression. In contrast to NFATc2, however, ChIP experiments demonstrated that T-bet does not bind the IL-21 proximal promoter in Th1 or Th2 cells.

Interestingly, NFATc2 only bound the IL-21 promoter in Th2 cells (and in T-bet$^{-/-}$ Th1 cells) but not in T-bet expressing Th1 cells, which led us to examine the effects of T-bet on NFATc2 activity. Reporter-gene assays demonstrated that T-bet impairs NFATc2-mediated activation of not only the IL-21 promoter, but also a basic NFAT promoter lacking T-bet binding sites. In addition, we found that T-bet and NFATc2 physically interact. Taken together, these data suggest a previously uncharacterized mechanism of Th cell subset regulation where T-bet can bind to and prevent NFATc2 binding to target promoters, such as IL-21, in Th1 cells. However, it is also possible that T-bet can recruit NFAT to an appropriate regulatory region if there are coordinate T-bet and NFAT sites present, such as in the IFN-γ promoter, resulting in a positive effect on transcription.

There is already evidence to support such a model of T-bet/NFATc2 interaction. For example, NFATc2 only binds the IL-4 promoter in Th2 cells, which do not express T-bet, whereas NFATc2 binds the IFN-γ promoter in T-bet-expressing Th1 cells (9, 24). It has also been demonstrated that NFATc2 no longer binds the IFN-γ promoter in T-bet$^{-/-}$ Th1 cells, even though there is still a stimulation-dependent increase of histone acetylation at this site, indicating the presence of "open" chromatin (24). There is further evidence of other IFN-γ regulatory regions, specifically a 5′ CNS, containing clustered T-bet, NFAT, and AP-1 sites. T-bet and NFATc2 together synergistically activate an IFN-γ reporter gene containing the 5′ CNS. It was also found that NFATc2 selectively binds the 5′ CNS only in Th1 cells, as does T-bet, in ChIP assays (28). Thus, our model of T-bet and NFATc2 interaction suggests two distinct outcomes depending on the context of the promoter. In one case, such as the IFN-γ gene, T-bet and NFATc2 cooperatively bind and promote transcriptional regulatory elements. In the second case, such as the IL-21 promoter, where coordinate T-bet and NFAT sites do not exist, T-bet prevents NFATc2 from binding and activating its regulatory elements on its own. While it is possible that T-bet may bind directly to other regions of the IL-21 gene to inhibit transcription, repression by T-bet does occur in the context of the proximal promoter, which lacks T-bet binding sites. Thus, the Th1-restricted expression of T-bet provides a mechanism for the regulation of subset-restricted NFATc2-activated genes.

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